HOST RESPONSE TO INFECTION

Reduced bactericidal activity against Staphylococcus aureus and Pseudomonas aeruginosa of blood neutrophils from patients with early adult respiratory distress syndrome

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This study investigated the bactericidal capability of circulating neutrophils from blunt trauma patients admitted to an Intensive Care Unit against Staphylococcus aureus and Pseudomonas aeruginosa. Among those patients, two groups were considered and compared: patients who developed adult respiratory distress syndrome (ARDS) and patients who developed only pneumonia. Peripheral blood samples were drawn as soon as a diagnosis of pneumonia or ARDS was made, followed by the isolation of neutrophil cells and assessment of bacteria phagocytosis and killing. The results demonstrated that in patients with ARDS, phagocytosis and killing efficiency were significantly impaired in comparison with patients with pneumonia and healthy controls. A possible dysregulation of reactive oxygen species production involving the release of humoral mediators in early ARDS may be involved.

Introduction

It is well known that the non-specific immune response to bacterial infection is mediated primarily by neutrophil polymorphonuclear leucocytes (PMNLs), which represent the ‘professional’ phagocytic cells deployed to eradicate invading micro-organisms. PMNLs are also involved in the pathogenesis of many inflammatory syndromes including the adult respiratory distress syndrome (ARDS) [1, 2]. Despite many advances in supportive care, ARDS still has a high mortality rate and there is clear evidence that ARDS patients usually die of infection, particularly lower respiratory tract infections [3]. Patients with ARDS have been shown to have alterations in the function of circulating neutrophils or those in the lungs, or both, and such a dysfunction could contribute to the enhanced risk of infection in these patients [4, 5].

The present study sought to evaluate the bactericidal efficiency of circulating neutrophils from patients admitted to an Intensive Care Unit (ICU). Patients with blunt chest trauma who developed pneumonia or ARDS were compared, as soon as a diagnosis of pneumonia or ARDS had been made.

Materials and methods

Study design

The study was performed after approval by the institutional ethics committee and informed consent was obtained from each patient or their next of kin. Sixty subjects admitted to an Intensive Care Unit (ICU) with blunt chest trauma were enrolled over a 28-month period (Jan. 1998–Jan. 2000). Patients were excluded from the study for the following reasons: need for antibiotic therapy; presence of infection on admission; age <18 years; suspected pregnancy or post-partum state; immunosuppressed state (i.e., treatment with steroids, bones marrow or organ transplant recipients, haematological malignancy and AIDS); medical condition considered to be irreversible.

The acute physiology and chronic health evaluation (APACHE II) score was employed to determine the initial severity of illness [6]. Two groups of patient were investigated: those who developed pneumonia during hospitalisation and those who developed ARDS. The following criteria for clinical diagnosis of
pneumonia were used: radiographic evidence of a new and persistent pulmonary infiltrate (other than those of non-infectious origin), fever (>38°C), leucocytosis (≥ 12 × 10⁹/L) and purulent respiratory secretions. ARDS was defined by the presence of classic criteria revised by the American-European Consensus Conference [7] as follows: a compatible underlying disease, a pulmonary artery occlusion pressure of <18 mmHg during mechanical ventilation and bilateral lung opacities. On the diagnosis of pneumonia or ARDS, blood samples were withdrawn to isolate PMNLs and to measure adhesivity, phagocytosis and killing of *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 6538). These organisms were chosen because they represented the species most frequently isolated from broncho-alveolar lavages (BALs) of patients admitted to the ICU. Ten healthy controls were selected and compared with the population under study. These controls were matched by age, sex and seasonal time of enrolment.

**Preparation of human PMNLs**

PMNLs were collected from healthy donors and from the patients by Boyum’s technique [8]. PMNLs were used within 1 h of isolation. Cell preparations were >95% neutrophils by Diff-Quick staining (Baxter Scientific Products, Miami, FL, USA) and were >96% viable in trypan blue exclusion tests.

**Labelling and opsonisation**

Attachment and ingestion of bacteria by PMNLs were assessed by direct visualisation with a modification of a previously described fluorescence microscopy method [9]. For each phagocytosis assay, an overnight culture of micro-organisms in Brain Heart Infusion (BHI; Merck Darmstadt Germany) broth was diluted 1 in 100 in fresh BHI broth and grown to mid-log phase. Bacterial density was adjusted spectrophotometrically to a concentration of 2 × 10⁸ cfu/mL. Bacteria were labelled by incubation with fluorescein isothiocyanate (FITC; Sigma) 0.1% in 50 mM sodium carbonate buffer, pH 9.6, for 30 min at 37°C in the dark. Labelled organisms were washed twice and suspended in Hank’s Balanced Salts Solution (HBSS) without Ca²⁺ and Mg²⁺. The FITC-labelled bacteria were then opsonised by incubation with pooled normal human serum 10% in HBSS at 37°C for 15 min with rotation. After opsonisation, bacterial cells were pelleted, washed and resuspended in HBSS.

**Phagocytosis**

Each phagocytic mixture contained 0.5 mL of opsonised fluorescein-labelled bacteria (2 × 10⁷ cfu/mL) and 0.5 mL of PMNLs (2 × 10⁶/mL) in HBSS plus 1 mM Ca²⁺ and 1 mM Mg²⁺. The ratio of bacteria to cells was 10 to 1. The tube was rotated for 30 min at 37°C, then 200-mL samples were removed. In experiments with *S. aureus*, the mixtures were treated with lysostaphin 10 mg/L for 5 min at 4°C to kill any residual extracellular bacteria. Incubation at 4°C prevented any effect of lysostaphin on intracellular *S. aureus*, as lysostaphin does not enter cells at low temperatures but does eliminate extracellular bacteria. Ten mL of ethidium bromide 0.1% were added to each phagocytic mixture to a final concentration of 50 µg/mL. Then 10 µL of the mixture were placed on a glass slide, overlaid with a coverslip and examined, within 20 min, with a fluorescence microscope with a 520-nm FITC filter under oil immersion (magnification, ×1000). Surface-attached or extracellular bacteria appeared orange, whereas ingested micro-organisms showed a rim of intense apple-green fluorescence without any orange staining. At least 20 PMNLs per sample were examined to evaluate the number of attached or ingested organism per cell [10]. Intracellular viable bacteria were determined by standard colony counts (cfu/mL).

**Killing**

Bacteria suspended in HBSS and PMNLs at a ratio of 10:1 were incubated in a water bath with agitation at 37°C (in polypropylene vials) for 3 h. Bacteria without PMNLs were also checked to determine bacterial growth and to prevent spontaneous agglutination during the incubation time. At 60, 120 and 180 min samples were taken in triplicate, mixed with distilled water to lyse the PMNLs and, after 10-fold serial dilutions, suspended in molten BHI agar for enumeration of cfu/mL after 24 h (determination of the number of intracellular viable bacteria) Bacterial multiplication in the medium over a 30-min period was eliminated as a factor by counting the bacterial inoculum incubated in the medium in the absence of PMNLs: no significant increase occurred during the 30-min period.

**Assessment of bacterial phagocytosis, killing and association to PMNLs**

Phagocytosis was expressed as the decrease in the initial number of viable extracellular bacteria in the inoculum by the following formula: \(\Phi(t) = N_0 - N_t\) in which \(N_0 = \) Phagocytosis at time \(t\), \(N_t = \) the number of viable bacteria incubated in medium for 30 min at 37°C in the absence of PMNLs and \(N_t = \) the number of viable extracellular bacteria in the presence of PMNLs after incubation for 30 min. Bacterial killing was calculated by the following equation: \(K(t) = \Phi(t) - V(t)\) in which \(K(t) = \) killing at time \(t, \Phi(t) = \) phagocytosis at time \(t\) and \(V(t) = \) VB is the number of viable intracellular bacteria at time \(t\). Bacterial association to PMNLs was evaluated by examining at least 200 PMNLs and determining the percentage of cells that had two or more associated bacteria.
Detection of PMNL receptors

Monoclonal antibodies (MAbs) (Becton-Dickinson, Milan, Italy) against the PMNL receptors CD11 and CD16 were used. The former is specifically directed towards the complement fraction C3b, whereas the latter is specific for the Fc component of the immunoglobulins. Heparinised blood (20-ml amounts) from the patients was added to two different tubes with 5 ml of MAbs – anti-CD11 conjugated with fluorescein (FITC) and anti-CD16 conjugated with phycoerythrin (PE), respectively. Controls were run with blood from healthy donors. The tubes were incubated for 20 min in the dark, then 1 ml of a lysis solution was added to destroy the red cells. After further incubation for 10 min in the dark, the suspensions were centrifuged for 5 min at 380 g. The pellets were washed with 1 ml of PBS and centrifuged for 5 min at 380 g. After removing the supernates, the pellets were suspended again in 250 ml of PBS. Flow cytometric readings were taken after careful mixing of the suspensions. Each suspension was aspirated and then passed through a laser beam emitting either red (for CD16) or green (for CD11) fluorescence, the intensity of which corresponded to the expression of each receptor. The results were reported as the percentage of PMNLs expressing the two receptors (semi-quantitative results).

Statistical analysis

Data are expressed as mean and SD. Wilcoxon’s rank sum test was used for comparison of values between pneumonia patients and ARDS patients, with a significance threshold of p < 0.05. The Mann-Whitney test was employed to compare the patient characteristics.

Results

Patients

Of the 60 patients enrolled at the beginning of the study, 38 patients were followed: 20 who developed pneumonia during hospitalisation and 18 who developed ARDS. Table 1 shows the demographic data and the APACHE II scores, as well as the mortality rate of pneumonia patients and ARDS patients. There was no significant difference between the two groups of subjects. Mechanical ventilation was performed with a volume-controlled ventilator (Servo 9000 C; Siemens Elema Solna, Sweden) and continuous analgesic sedation with midazolam and fentanyl was maintained. Ventilator settings, level of positive end-expiratory pressure and fractional inspired oxygen (FIO2) were adjusted as necessary. All patients were treated with supportive fluid infusion, histamine-2-inhibitor and, as needed, cardiac glycosides. The mean hospital stay was 12 (SD3) days.

Bacterial association with human PMNLs

Table 2 shows the mean ingestion of P aeruginosa and S. aureus by PMNLs from pneumonia patients and ARDS patients compared with PMNLs from healthy controls. No significant difference in percentage uptake was found either in the three groups of patients or in the different bacterial targets: in fact, a mean value of ingestion equal to 36.6% (range 28–45) was observed for the three groups of patients.

Phagocytosis and killing

A correlation of 0.9 between cfu and fluorescence values established the validity of both microbiological and microscopic assays in the assessment of intracellu-

Table 1. Demographic data, APACHE II score and mortality rate of patients with pneumonia or ARDS

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age</th>
<th>Sex (M/F)</th>
<th>APACHE II score</th>
<th>Mortality rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia patients</td>
<td>20</td>
<td>59 (12)</td>
<td>11.9</td>
<td>19 (2)</td>
<td>29</td>
</tr>
<tr>
<td>ARDS patients</td>
<td>18</td>
<td>59 (17)</td>
<td>7/11</td>
<td>23 (3)</td>
<td>43</td>
</tr>
</tbody>
</table>

*Data are expressed as mean (SD).

Table 2. Ingestion of P aeruginosa and S. aureus by PMNLs from each patient group.

<table>
<thead>
<tr>
<th>Source of PMNLs</th>
<th>n</th>
<th>P. aeruginosa</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>10</td>
<td>45.0 (12.5)</td>
<td>36.2 (9.7)</td>
</tr>
<tr>
<td>Pneumonia patients</td>
<td>20</td>
<td>40.1 (9.8)</td>
<td>32.5 (8.9)</td>
</tr>
<tr>
<td>ARDS patients</td>
<td>18</td>
<td>38.2 (7.9)</td>
<td>28.0 (8.7)</td>
</tr>
</tbody>
</table>

Data are expressed as mean (SD).
lar viable bacteria. The results obtained with *S. aureus* in the study population are given in Fig. 1. During a 30-min incubation of 2.0 × 10³ cfu of bacteria/ml with PMNLs (2.0 × 10³), the parameters of phagocytosis and killing were calculated by using the cfu and the fluorescence assay. When the numbers of *S. aureus* phagocytosed and killed in healthy controls were used as base-line values, valid comparisons could be made; the numbers of *S. aureus* phagocytosed and killed by healthy controls were calculated as 3.75 (SD 0.34) × 10⁶ and 3.25 (SD 0.30) × 10⁵ cfu/ml, respectively. In ARDS patients, *S. aureus* was less efficiently phagocytosed and killed during the same 30-min, period – 1.50 (SD 0.24) × 10⁶ and 1.25 (SD 0.20) × 10⁵ cfu/ml. The decrease in phagocytosis and killing in this group of patients was statistically significant compared with healthy controls and pneumonia patients (p < 0.05), in which the bacteria phagocytosed and killed were 3 (SD 0.25) × 10⁶ and 2.75 (SD 0.20) × 10⁵cfu/ml, respectively. The study also demonstrated that *S. aureus* was more readily eliminated in the controls than in the other two groups. The proportion of killed to surviving bacteria phagocytosed by PMNLs during a 30-min period was higher in the controls (5.0) than in pneumonia patients (3.05) or ARDS patients (1.95) (data not shown). The data obtained for *P. aeruginosa* were similar to those obtained for *S. aureus* (Fig. 2). Again, it was evident that the difference in phagocytosis and killing between the ARDS patients and healthy controls was statistically significant (p < 0.05), whereas no significant difference was observed between the controls and pneumonia patients. *P. aeruginosa* appeared to be less amenable to phagocytosis and intracellular killing than *S. aureus*. In fact, the numbers of phagocytosed and killed *P. aeruginosa* were lower than the correspondent values of *S. aureus*: 1.5 (SD 0.24) × 10³ and 1.25 (SD 0.20) × 10² cfu/ml in healthy controls; 8 (SD 0.25) × 10³ and 6 (SD 0.18) × 10³ cfu/ml for pneumonia patients; 3.20 (SD 0.8) × 10³ and 3 (SD 0.5) × 10³ cfu/ml for ARDS patients, respectively.

**Analysis of PMNL receptors**

The measurement of PMNL receptors is reported in Table 3, which shows the mean values for both groups of patients (with ARDS and with pneumonia) and the controls. No difference was evident among the three groups; in fact, the percentages of CD11 and CD16 were e. 99% and 1.5%, respectively, in all groups.

**Discussion**

The data from the present study indicated that in blunt trauma patients, those who developed ARDS early exhibited an alteration of circulating PMNLs in terms of both reduced phagocytosis and decreased killing of bacterial strains such as *S. aureus* and *P. aeruginosa*. This defect appears significantly obvious if compared with the results obtained in blunt trauma patients who developed only pneumonia.

By contrast, no differences in bacterial ingestion by PMNLs was found in the two groups of patients nor in the controls. This observation was sustained by the finding of a similar percentage of PMNL receptors in the population under study. Indeed, it was observed that

![Fig. 1. *S. aureus* (cfu/ml) phagocytosed (□) and killed (■) by PMNLs from healthy controls (I), pneumonia patients (II) and ARDS patients (III). Data are expressed as mean and SD. *p < 0.05 versus results for controls and pneumonia patients.](image-url)
PMNs from all groups expressed similar levels of receptors such as CD11 and CD16, with c. 99% and 1.5% for CD11 and CD16, respectively.

It is conceivable that the reduced bactericidal capacity of the blood PMNs found in ARDS patients could be attributable to an altered cell function, namely an impairment of respiratory burst activity, because of the central role played by the production of reactive oxygen species (ROS) [12].

Previous studies have shown PMNs from ARDS patients show functional and metabolic alterations [13,14], but the literature is contradictory regarding ROS generation. Some authors described an enhanced production while others found decreased release of superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$). For example, Rivkind and co-workers observed activation of the neutrophil oxidative burst in circulating PMNs from ARDS patients as assessed by superoxide anion secretion [15]. However, Parson et al. reported that PMNs from patients with ARDS produced less superoxide than did cells from normal subjects when primed with lipopolysaccharide (LPS) or stimulated with N-formyl-methionyl-leucine-phenylalanine [16].

Chollet-Martin et al. found that circulating PMNs from ARDS patients exhibited a bimodal response of H$_2$O$_2$ production in flow cytometry studies [17]. This observation confirmed the functional heterogeneity of PMNs described elsewhere [18] and could explain in part the discordant results obtained so far.

An impairment of both bactericidal and oxidative burst characteristics was noticed not only in circulating but also in alveolar cells isolated from lungs by broncho-alveolar lavage (BAL) [19]. Chollet-Martin et al. showed dysregulation of activity, and this could explain, in the opinion of the authors, the increased susceptibility to bacterial infections in ARDS patients [20]. In line with this finding, other studies demonstrated a reduced phagocytosis of Escherichia coli by alveolar macrophages during endotoxaemia and a down-regulation of ROS production by the same macrophages was noted during P. aeruginosa bacteremia in an experimental animal model [21, 22].
Unfortunately, in the present study the production of reactive oxygen metabolites was not measured. Never- theless, on the basis of the data reported, the results seem to support indirectly the theory of a down-regulation of the ROS secretion by circulating PMNLs in early ARDS. The exact mechanism causing this defect is still not clearly understood; some studies point out the main involvement of humoral mediators and the interaction between PMNLs and cytokines [23, 24]. High levels of tumour necrosis factor-α (TNF-α), as well as of interleukin-6 (IL-6) and IL-8, have been found in plasma and in BAL of ARDS patients [25, 26]. These compounds are able to activate PMNL function in vivo, accounting for the correlation observed between their enhanced systemic concentration and the expression of CD11b and CD62L receptors [20].

Yuan et al. observed in vitro the effects of some pro-inflammatory cytokines including granulocyte-macro- phage colony-stimulating factors (GM-CSF), IL-2, IL6, IL-1α, IL-1β and IFN-β on intracellular oxidative production in normal PMNLs [27]. Whereas GM-CSF had a marked influence on H2O2 release, other mediators such as IL-1α were less effective. On the contrary, IL-10, an immunomodulatory cytokine which shows an anti-inflammatory effect, would inhibit the ROS generation by PMNLs [28].

In early ARDS, pathways could be triggered inducing the release of humoral mediators which down-regulate the PMNL bacterial activity by interfering with ROS generation. However, to test and confirm this hypothesis, further studies, addressing the interaction between systemic inflammatory mediators and the bactericidal function of PMNLs in ARDS, will be required.

References

8. Boyum A. Isolation of lymphocytes, granulocytes and macro-